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High Antibody Levels to Prothrombin Imply a Risk of Deep Venous Thrombosis and Pulmonary Embolism in Middle-aged Men

A Nested Case-control Study

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Summary

Antibodies against phospholipid-binding plasma proteins, such as β_2-glycoprotein I (β_2-GPI) and prothrombin, are associated with thromboembolic events in patients with systemic lupus erythematosus and also in subjects with no evident underlying diseases. We wanted to examine whether increased levels of antibodies to negatively-charged phospholipids (cardiolipin), to phospholipid-binding plasma proteins β_2-GPI and prothrombin and to oxidised low-density lipoprotein (LDL) were associated with risk of deep venous thrombosis or pulmonary embolism in subjects with no previous thrombosis. The antibodies were measured in stored serum samples from 265 cases of deep venous thrombosis of the lower extremity or pulmonary embolism occurring during a median follow-up of about 7 years and from 265 individually matched controls. The study subjects were middle-aged men participating in a cancer prevention trial of alpha-tocopherol and beta-carotene and the cases of thromboembolic events were identified from nationwide Hospital Discharge Register.

The risk for thrombotic events was significantly increased only in relation to antiprothrombin antibodies. As adjusted for body mass index, number of daily cigarettes and history of chronic bronchitis, myocardial infarction and heart failure at baseline, the odds ratio per one unit of antibody was 6.56 (95% confidence interval 1.73-25.0). The seven highest individual optical density-unit values of antiprothrombin antibodies were all confined to subjects with thromboembolic episodes.

In conclusion, the present nested case-control study showed that high autoantibody levels against prothrombin implied a risk of deep venous thrombosis and pulmonary embolism and could be involved in the development of the thrombotic processes.

Introduction

A heterogeneous group of immunoglobulins binding to negatively charged phospholipids, phospholipid-protein complexes, or directly to certain phospholipid-binding plasma proteins are traditionally called as antiphospholipid (aPL) antibodies (1-5, for review, see 6). These antibodies are detected by solid-phase immunoassays using as antigens anionic phospholipids, such as cardiolipin or phosphatidyl serine, and, by lupus anticoagulant test which measures prolongation of phospholipid-dependent coagulation time. Antibodies found in association with infections seem to be directed against the phospholipid molecules themselves and thus are considered to represent genuine aPL antibodies. The majority of autoimmune-type antibodies detected by solid-phase immunoassays are dependent on the presence of β_2-glycoprotein I (β_2-GPI) in the test system (2, 3), while in lupus anticoagulant assays, several plasma proteins, such as β_2-GPI, prothrombin, and also protein C or protein S can be the antigenic targets (7-11). Antibodies binding to oxidised low-density lipoprotein (LDL) are also considered aPL antibodies because LDL contains both phospholipids and lipid-binding protein apolipoprotein B (12, 13).

The antiphospholipid syndrome, characterized by the presence of aPL antibodies in association with arterial and venous thromboses, recurrent fetal loss and thrombocytopenia, was first described in patients with systemic lupus erythematosus (SLE) but it has been later observed that thrombotic episodes may also occur in patients with these antibodies but without clinical manifestations of SLE (14). Two studies on patients without any evidence of SLE have revealed a significant association between the presence of lupus anticoagulant and venous thrombosis and pulmonary embolism (15, 16). In contrast, findings concerning antibodies against cardiolipin as measured by solid-phase immunoassays have been controversial. A nested case-control study in a prospective cohort of healthy adult men by Ginsburg et al. (17) found an association between anticardiolipin antibodies and deep vein thrombosis, whereas retrospective studies by Bongard et al. (18) and Ginsberg et al. (15) failed to do so.

Availability of a large cohort of middle-aged men, followed up for an average of 7 years (19), prompted us to study whether antibodies to plasma phospholipid-binding proteins β_2-GPI and prothrombin were associated with increased risk of venous thromboses and pulmonary embolism. For comparison, antibodies to cardiolipin and to oxidised LDL were also investigated.

Materials and Methods

Study Population

This study was carried out within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (ATBC Study), a randomized, double-blind, placebo-controlled prevention trial examining whether supplementation with alpha-tocopherol, beta-carotene, or both would reduce the incidence of lung cancer in male smokers. The rationale, design and methods, and the characteristics of the participants have been described in detail elsewhere (19).

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The participants of the ATBC Study were male smokers (five or more cigarettes per day at entry) aged 50 to 69 years who were recruited from the total male population of this age group residing in 14 local study areas in southwestern Finland (n = 290,406). Potential participants were excluded if they had a history of cancer or serious disease limiting ability to participate, or were taking supplements of vitamin E, vitamin A, or beta-carotene in excess of predefined doses, or were being treated with anticoagulant agents. Eligibility was assessed during two baseline visits after which the participants (n = 29,133) were randomly assigned to one of the four supplementation regimens: alpha-tocopherol 50 mg per day, alpha-tocopherol 50 mg and beta-carotene 20 mg per day, beta-carotene 20 mg per day, or placebo. Data on medical, dietary and smoking histories, and general background characteristics were obtained at study entry. Height and weight were also measured, and a blood sample was drawn and serum stored at –70° C. All participants provided written informed consent before randomization.

Participants were recruited between 1985 and 1988 and supplementation continued for a median of 6.1 years until trial closure (April 30, 1993), and follow-up was continued threreafter.

The end points of the present study were deep venous thrombosis of the lower extremity and pulmonary embolism. Only the first event after randomization of either entity was registered as an end point. In Finland, the diagnosis of deep vein thrombosis is based on venography and that of pulmonary embolism on perfusion lung scanning. Nearly all patients are treated as inpatients.

Cases were identified from the nationwide Hospital Discharge Register using the unique personal identity code for record linkage. The register was established in 1967, and since then, 282 ATBC Study participants had suffered from venous thrombosis in the lower extremity or pulmonary embolism prior to study randomization, and thus were excluded from the study described here. In addition, 17 men reported at baseline a history of deep vein thrombosis in the lower extremity diagnosed by a physician and were also excluded. After randomization, 265 men were diagnosed with their first deep vein thrombosis or pulmonary embolism by the end of 1993 (median time from randomization to diagnosis was 3.6 years, range from 0.0 to 8.4 years). Among these subjects, the diagnosis was lower extremity thrombosis in 118 instances, pulmonary embolism in 132 instances, and both diagnoses for 15 cases.

For each case, one control was selected from those participants who were at risk at the time of the diagnosis of deep venous thrombosis or pulmonary embolism of the case and had a baseline serum sample available. The controls were further matched for age (± 1 year), supplementation group, study area, and timing of the baseline blood sampling (± 14 days). Thus 265 eligible case-control pairs were established. Selected base-line characteristics of the study population are shown in Table 1.

Purification of β_2-glycoprotein I

β_2-GPI was purified from normal human plasma as described in detail previously (20). The plasma was treated with 3% perchloric acid to precipitate serum proteins other than β_2-GPI. The precipitate was removed by centrifugation, the supernatant was collected and dialysed against 0.03 M NaCl in phosphate buffered saline (PBS), pH 8.0. The solution was then chromatographed on a Heparin-Sepharose CL-6B column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and the proteins were eluted with 0.03 M, 0.15 M and 0.35 M NaCl in PBS, pH 8.0. Fractions concentrated with nitrogen stream were then run through an FPLC reversed-phase ProRPC HR 5/10 column (Pharmacia) using a linear gradient of acetonitrile (10-60%, 40 min) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. β_2-GPI eluted at 23-27% acetonitrile concentration. The fractions were dried in a vacuum centrifuge and redissolved into PBS. The protein concentration was measured by the Lowry method and the absence of other proteins in the preparation was assured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1 Selected characteristics of study population of 265 matched pairs at baseline. Pair differences were tested with t-test for continuous and with χ^2-test for categorial variables

	Cases[1)]	Controls[1)]	p
Age (years)	59.3 (5.2)	59.3 (5.2)	0.167
Body mass index	27.0 (4.3)	26.4 (4.0)	0.096
Number of daily cigarettes	21.4 (8.5)	19.8 (8.8)	0.029
Chronic bronchitis (%)	12.5	6.8	0.039
Myocardial infarction (%)	12.1	5.3	0.009
Heart failure (%)	7.5	3.0	0.033

[1)] Figures in parentheses denote standard deviation.

Anti-prothrombin ELISA

A method described in detail earlier was used (20). Irradiated polystyrene plates (Combiplate® Enhanced Binding, Labsystems, Helsinki, Finland) were coated with human prothrombin (Sigma Chemical Co., St. Louis, MO) at a concentration of 10 µg/ml in PBS, 100 µl per well and incubated overnight at +4° C. The plates were washed with 0.05% Tween-PBS before blocking with 3% gelatin in PBS for 1 h at room temperature. Serum samples were diluted 1:50 in 0.3% gelatin-PBS and incubated for 1.5 h at room temperature. Alkaline phosphatase-conjugated rabbit $F(ab')_2$ fragment anti-human IgG antibodies (Jackson Immunoresearch Laboratories, West Grove, PA), diluted 1:2000 in 0.2% human serum albumin (HSA)-PBS, was added and incubated for 1 h at room temperature. P-nitrophenyl phosphate (Sigma), 1 mg/ml in carbonate buffer, pH 9.8, was used as a substrate and incubated for 30 min at room temperature. The absorbance was measured by an optical reader at 405 nm and recorded as optical density (OD) units. The assay was standardized by using two known positive and two known negative serum samples on each plate. The mean intra-assay variation for the method was 4.8% and interassay variation was 10.2%.

Anti-β_2-glycoprotein ELISA

The assay was performed as was the anti-prothrombin ELISA with the following exceptions: the concentration of β_2-GPI used for coating was 5 µg/ml and the samples were diluted 1:200. The intra-assay precision of the method was 4.6% and inter-assay precision 16.8%.

Anti-cardiolipin ELISA

IgG class anti-cardiolipin antibodies were detected according to a previously described ELISA method with the exception that 10% bovine serum was used as the blocking solution (12). The mean OD-value + 2 standard deviations of the control subjects was 0.37, which corresponded to about 30 IgG antiphospholipid antibody (GPL) units/ml.

Antibodies to Oxidised LDL

IgG class antibodies to malondialdehyde-modified LDL were measured using an ELISA method described previously (12).

Statistical Methods

Differences between cases and controls in the antibody levels and values of continuous background characteristics were compared with the paired t-test. Differences regarding categorical variables were compared with chi-square tests. Conditional logistic regression that took into account pair matching was used in modeling (21). Results are presented as odds ratios (OR) per one unit for the antibodies as continuous variables and adjusted for body mass index, number of daily cigarettes, and history of chronic bronchitis, myocardial infarction and heart failure.

Results

Mean antiprothrombin antibody level was significantly higher in the men with thrombotic events than in the controls (0.272 OD-units versus 0.226 OD-units), whereas no significant differences were noted in the antibody levels against cardiolipin, β_2-GPI and oxidised LDL

Antibody level to:	Cases	Controls	Odds ratio	**Adjusted odds ratio***
cardiolipin	0.18 (0.21)	0.17 (0.15)	1.57 (0.57, 4.37)	**1.18 (0.43, 3.19)**
β_2-glycoprotein I	0.33 (0.15)	0.31 (0.14)	1.86 (0.46, 7.59)	**1.47 (0.34, 6.38)**
oxidised LDL	0.22 (0.12)	0.22 (0.12)	1.23 (0.25, 6.13)	**1.08 (0.21, 5.48)**
prothrombin	0.27 (0.27)	0.23 (0.12)	3.71 (1.12, 12.4)	**6.54 (1.73, 25.0)**

* Adjusted for body mass index, number of daily cigarettes and history of chronic bronchitis, myocardial infarction and heart failure at baseline.

Table 2 Antibodies (optical density values) against cardiolipin, β_2-glycoprotein I, oxidised LDL and prothrombin, means and standard deviations. Odds ratios cases vs. controls from conditional logistic regression with 95% confidence limits

(Table 2). The risk for thrombotic events was significantly increased only in relation to antiprothrombin antibodies (odds ratio 3.71, 95% confidence interval 1.12-12.37; odds ratio as adjusted for body mass index, number of daily cigarettes and history of chronic bronchitis, myocardial infarction and heart failure at baseline, 6.56, 95% confidence interval 1.73-25.0; Table 2). Figure 1 shows that the distribution of antiprothrombin antibody levels among the cases was different from that of the controls. The seven highest individual OD-unit values of antiprothrombin antibodies were all confined to subjects with thromboembolic episodes. If these seven cases were excluded the distributions were virtually identical in cases and in controls. Data on antibody levels and short clinical histories of the 7 cases are compiled in Table 3. It can be noted that antibody levels against cardiolipin, β_2-GPI and oxidised LDL were not elevated in any of these cases.

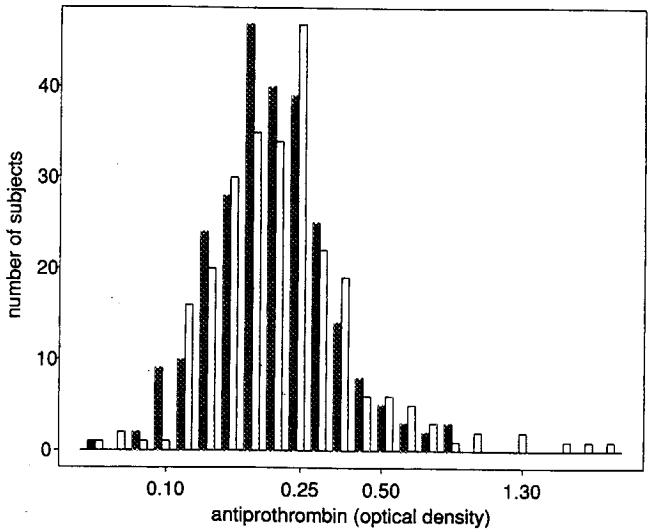

Fig. 1 Distribution of antibody levels against prothrombin in cases (open columns) and in controls (hatched columns)

Discussion

High autoantibody levels against prothrombin implied a risk of deep venous thrombosis and pulmonary embolism in the present nested case-control study of a cohort of middle-aged men. We have recently shown that elevated levels of antibodies to prothrombin predicted myocardial infarction in a prospective cohort of middle-aged men (22). In that study, the difference between cases and controls emerged over a wide range of antibody levels. In the study described here, a number of subjects had high antibody levels against prothrombin, comparable to those seen in SLE (20), that clearly exceeded the highest levels observed in controls; apart from these subjects, the distribution of antibody levels in cases ands controls were very similar. Since the study design did not include the evaluation of lupus anticoagulant, we cannot establish whether subjects with high antibody levels against prothrombin were positive for the lupus anticoagulant antibodies although this is a reasonable possibility (23). With regard to antibodies against β_2-GPI the levels in cases were slightly higher than in controls. The difference, however, did not reach statistical significance. Antibody levels against cardiolipin and against oxidised LDL were not different in cases and in controls.

Most (10, 20, 23, 24) but not all (25) studies have shown that occurrence of antibodies to prothrombin is associated with thrombosis in patients with SLE and primary antiphospholipid syndrome. Yet antiprothrombin antibodies do not show a close correlation with antibodies to β_2-GPI. Patients with SLE also frequently have elevated levels of antibodies against oxidised LDL (12, 13, 26) but these antibodies seem to be associated solely with atherosclerotic conditions, such as progression of carotid atherosclerosis (27, 28) and myocardial infarction (29). Also in studies on patients with SLE, antibodies to oxidised LDL were found to be elevated only in patients with arterial thrombosis (30) whereas in patients with venous thrombosis antibodies to oxidised LDL were not elevated (30, 31).

Arterial thrombosis seldom occurs without underlying atherosclerosis. In contrast, venous thromboembolism is not infrequently seen in subjects without any evidence of atherosclerosis. Somewhat curiously, so-called aPL antibodies are associated with both arterial and venous thrombosis, although the risk factors for these two conditions are not the same. It seems that aPL antibodies can be divided into subgroups according to their specificity and their possible disease associations

Case no	anti-prothrombin	anti-cardiolipin [1)]	anti-β_2-glycoprotein I [1)]	anti-oxidised LDL [1)]	remarks
1	0.895	0.100	0.310	0.069	complicated fibular fracture 4 months earlier
2	0.989	0.088	0.308	0.360	-
3	1.232	0.075	0.231	0.124	cerebral stroke one month earlier
4	1.273	0.058	0.228	0.120	several venous and arterial thromboses subsequently
5	1.962	0.161	0.357	0.313	malleolar fracture one month earlier
6	2.083	0.168	0.374	0.402	multiple myeloma diagnosed at same time as venous thrombosis
7	2.745	0.048	0.371	0.314	seropositive rheumatoid arthritis diagnosed two years earlier

[1)] None of the values exceeded mean + 2 standard deviations of the controls.

Table 3 Antibody levels (optical density values) and brief clinical histories of the seven cases with highest antiprothrombin antibody levels

(32). Whether or not these antibodies play pathogenic roles may depend on their affinity for physiologically relevant targets and the accessibility of the targets for the antibodies (5). Progress in the area will probably depend on the accurate classification of the aPL antibodies on the basis of the targets with which they interact. Advent of techniques to measure antibodies against different phospholipid-binding plasma proteins offer certain promise in this respect (4, 10, 33).

For the time being, little is known about the epitopes of the targets of antibodies binding to phospholipids or to phospholipid-binding plasma proteins but the possibility that some (as yet undefined) antibody specificities could have direct roles in the pathogenesis of thrombotic events is a reasonable possibility. It is generally acknowledged that patients with SLE have a wide array of autoantibodies and there is increasing evidence to support the contention that these autoantibodies are antigen-driven and that they are responsible for at least some of the clinical manifestations of the disease (34, 35). The initial autoantibody response can result in expansion of the antibody response to other epitopes to the self-antigen and, under certain circumstances, to other constituents of the complex in which the self-antigen is contained, an event referred to as epitope spreading (36).

In this kind of series of evolving epitopes it is likely that only a few will have relevance for the disease. E.g., evidence has been presented that there are 2 types of IgG anti-prothrombin antibodies that differ in their anticoagulant properties (37). Due to multitude of autoantibodies and wide variety of clinical manifestations it may be difficult to unravel in SLE patients the disease-relevant epitopes that have a correlation with specific disease features. On the other hand, immunopathologic pathways in a variety of "autoimmune" diseases, other than SLE, may turn out to be more simple.

Nearly all previous studies dealing with aPL antibodies and thrombosis have been retrospective and the possibility that thrombotic events promoted the development of these antibodies must be taken into account. Our observation in the present prospective study showing that high and only high autoantibody levels against prothrombin were associated with deep venous thrombosis and pulmonary embolism point out that these antibodies indeed could have been involved in the process. Localization of the relevant antigenic epitopes may offer further insight into the mechanisms.

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